Redesign of the Interior Hydrophilic Region of Mitochondrial Cytochrome *c* by Site-Directed Mutagenesis†

Anne M. Davies,‡ J. Guy Guillemette,§ Michael Smith,§ Colin Greenwood,‖ Andrew G. P. Thurgood,‡ A. Grant Mauk,*,§ and Geoffrey R. Moore*,‡

School of Chemical Sciences and School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, U.K., and Department of Biochemistry, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada

Received December 14, 1992; Revised Manuscript Received February 24, 1993

ABSTRACT: Heme propionate-7 in cytochrome *c* is an ionizable group located in a region of the protein that is inaccessible to bulk solvent. Electrostatic stabilization of this functional group appears to be achieved through interaction of heme propionate-7 with several amino acid residues that occur within hydrogen-bonding distance of it. To investigate the functional and spectroscopic roles of the amino acid residues that contribute to the immediate environment of heme propionate-7, the following variant forms of yeast (*Saccharomyces cerevisiae*) cytochrome *c* have been prepared and characterized by electrochemical and spectrochemical analyses: Arg38Ala, Tyr48Phe, Ala38Phe, Tyr48Phe/Trp59Phe, and Arg38Ala/Tyr48Phe/Trp59Phe. For each protein, the dependence of midpoint reduction potential and NMR spectrum on pH was determined, and the UV (250–450 nm) circular dichroic (CD) spectrum was measured. All of the variant proteins exhibited decreased reduction potentials with the greatest difference (–65 to –70 mV) exhibited by the multiply mutated proteins. The electrostatic properties of the variant proteins as reflected by the oxidation-state dependence of the His-39 pK_a value were similar to those of the wild-type protein. Previous indirect assignments of minima in the CD spectrum of cytochrome *c* at 282 and 289 nm to Trp-59 are confirmed by spectra of the variant cytochromes in which this residue is replaced by Phe. The present results establish that the electrochemical effects of eliminating hydrogen-bonding interactions with heme propionate-7 are not additive and that the functional modulation of cytochrome *c* through regulation of the heme propionate-7 dielectric environment involves a complex combination of solvation effects and electrostatic or hydrogen-bonding interactions.

The heme-containing electron-transfer cytochromes *c* fall into several structural groups distinguished by their tertiary structures and the nature of their heme axial ligands [see Moore and Pettigrew (1990) and references cited therein]. Mitochondrial cytochrome *c*, whose main function is to transfer electrons in the respiratory chain from complex III to complex IV [see Pettigrew and Moore (1987) and references cited therein], and the bacterial cytochromes c_2 are members of the class I structural group (Ambler et al., 1980). The main characteristics of this class are that the heme attachment site, Cys-X-Y-Cys-His, occurs toward the N-terminal end of the protein, usually between residues 10 and 20, and a Met residue located approximately three-quarters of the way along the chain toward the C-terminal end acts as the sixth iron ligand. To date, the amino acid sequences of 96 mitochondrial cytochromes *c* and 20 cytochromes c_2 have been reported [for a recent compilation, see Moore and Pettigrew (1990)]. In addition, the X-ray structures of four species of mitochondrial cytochromes *c* (Takano & Dickerson, 1981a,b; Ochi et al., 1983; Bushnell et al., 1990; Louie & Brayer, 1990) and two species of cytochromes c_2 (Salemme et al., 1973; Benning et al., 1991) have been determined. These data show that there is a strong structural homology between cytochromes *c* and cytochromes c_2 with both sets of proteins having a common polypeptide fold that leads to the burial of the heme propionate substituents within the protein interior.

Comparison of the amino acid sequences of the mitochondrial cytochromes *c* reveals that there are 26 unvaried residues, out of 103–116, if the sequence of *Tetrahymena pyriformis* cytochrome *c* is excluded from the analysis [see Moore and Pettigrew (1990) and references cited therein]. The functions of some of these unvaried residues are known. For example, Cys-17, His-18, and Met-80 bind the heme group. Lys-72 and -79 are involved in intermolecular interactions, and glycines-1, -6, -29, -34, -41, -77, and -84 occur in type II β-bends or other regions of the structure where there is insufficient space for a side chain to be accommodated. However, the functions of some of the unvaried residues are not known. One such group of residues is the group interacting with the heme propionate-7[1] substituent: Asn-52, Arg-38, Tyr-48, and Trp-59.

The removal of the propionate groups from contact with bulk water leads to the establishment of a large internal hydrophilic region within the protein. At least two water molecules are located within this region in addition to the unvaried hydrophilic groups listed above. The fact that these unvaried groups interact with the heme suggests that they are important in creating the right environment for burial of the heme. This hypothesis appeared to be supported by studies on cytochromes c_2 [see Moore and Pettigrew (1990) and references cited therein]. None of the unvaried heme

† This research was supported by the Science and Engineering Research Council (SERC) through their Protein Engineering Initiative, by SERC support of the UEA Centre for Metalloprotein Spectroscopy and Biology through their Molecular Recognition Initiative, by NIH Grant GM33804 (A.G.M.), by MRC Grant MT-1706 (M.S.), and by NATO Travel Grant 0145/87. M.S. is a Career Investigator of the Medical Research Council of Canada.

‡ School of Chemical Sciences, University of East Anglia.
§ University of British Columbia.
‖ School of Biological Sciences, University of East Anglia.

[1] The Fisher nomenclature for heme substituents is used in this paper. The corresponding IUPAC designations for the groups discussed here are as follows (Fisher/IUPAC): heme propionate-7, C^{17}; heme methyl-8, C^{18}; protons of heme proprionate-7, H-$17^{1a,b}$.

0006-2960/93/0432-5431$04.00/0

propionate contact residues of mitochondrial cytochromes *c* are invariant in the cytochromes c_2, and these proteins have more variable heme properties than do the mitochondrial cytochromes *c*. For example, the heme propionate groups of mitochondrial cytochromes *c* do not ionize over the pH range 5–8, whereas in cytochromes c_2 lacking Arg-38 (they have either Gln or Asn at the equivalent position) heme propionate-7 ionizes with a pK_a in the range 6.0–7.5 and causes a significant perturbation of the heme reduction potential. Also, all mitochondrial cytochromes *c* and cytochromes c_2 having Trp-59 have a heme-linked ionization which results in the loss of the Met ligand over the pH range 8–11 whereas those cytochromes c_2 lacking Trp-59 (they have Ile in its place) lose their Met ligation with a pK_a ~7. Finally, whereas the reduction potentials of the mitochondrial cytochromes *c* at pH 7 are 260 ± 20 mV, those of the cytochromes c_2 range from 200 to 500 mV.

To investigate the role of the internal hydrophilic region of cytochrome *c*, we have used site-directed mutagenesis to introduce single, double, and triple substitutions at positions 38, 48, and 59. The results of this study, reported in the present paper, do not support the hypothesis that Arg-38, Tyr-48, and Trp-59 are unvaried because they determine the heme reduction potential or heme propionate ionization state.

EXPERIMENTAL PROCEDURES

Mutagenesis and Protein Isolation. A recombinant shuttle phagemid, pING4 (Inglis et al., 1991), was used for in vitro mutagenesis. The pING4 phagemid consists of the coding region and the upstream transcription control elements of yeast iso-1-cytochrome *c* (McNeil & Smith, 1986) which were cloned into the *Sma*I site of the pEMBLYe30 plasmid (Baldari & Cesareni, 1985). Site-directed mutagenesis was performed as previously described (Inglis et al., 1991) using the method that employed biological selection against the parental strand according to Kunkel (1985). The oligonucleotides used for mutagenesis were synthesized as described by Atkinson and Smith (1986), and the specific oligonucleotides employed in each case were as follows: 5′CCAGAGTGGGCGCCAAAGATA3′ (Arg38Ala); 5′GCATCTGTGAACGAATACC3′ (Tyr48Phe); 5′ATGTTATTTTCGTCGAACAACACGTT3′ (Trp59Phe). The Tyr48Phe/Trp59Phe and the Arg38Ala/Tyr48Phe/Trp59Phe variants were created by combining the appropriate oligonucleotides. All variants also possessed a second point mutation to change Cys-102 to Thr as discussed previously (Cutler et al., 1987); in this report, cytochrome *c* possessing only the Cys102Thr substitution is referred to as wild-type.

The recombinant plasmids isolated from the *Escherichia coli* JM101 (Messing, 1983) cells were used to transform the recipient yeast strain GM3C-2 by the poly(ethylene glycol) method as described by Bröker (1987). Strain GM3C-2 carries a deletion mutation that eliminates the gene for iso-1-cytochrome *c* as well as a point mutation that inactivates the gene for iso-2-cytochrome *c* (Faye et al., 1981). The mutated forms of the iso-1-cytochrome *c* protein were assayed for function by monitoring the growth of transformed yeast cells on a synthetic glycerol medium. The autonomous pING4 plasmids were rescued from yeast by the method described by Hoffman and Winston (1987) and then used to transform *E. coli* JF1754 cells (McNeil & Friesen, 1981). After passage through the endonuclease-deficient JF1754 cells, the methylated recombinant plasmids were isolated and used to transform *E. coli* JM101 cells. The entire coding region of the iso-1-cytochrome *c* gene was then sequenced to ensure that no further mutations had occurred during the manipulation of the plasmid.

Yeast iso-1-cytochromes *c* were expressed, isolated, and purified by the methods described previously (Cutler et al., 1987; Rafferty et al., 1990).

Electrochemical Measurements. Reduction potentials were measured by the method of mixtures with freshly prepared $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ as described by Pettigrew et al. (1975). As this method is particularly sensitive to ionic strength, the cytochrome was dialyzed extensively at 4 °C prior to use. The oxidation–reduction equilibrium of the cytochrome was monitored at 550 nm, and the final concentrations in the cuvette were as follows: 5×10^{-4} or 10^{-3} M $K_4Fe(CN)_6$, 1.7×10^{-6} or 3.4×10^{-6} M $K_3Fe(CN)_6$, 5×10^{-6} M cytochrome *c*, and 2×10^{-3} M buffer. The following buffers were used: sodium acetate (pH 4.5–5.8), sodium phosphate (pH 5.8–7.5), Tris-HCl (pH 7.5–8.8), glycine–NaOH (pH 8.8–9.3), and sodium borate (pH 9.3–10.7). In each case, the final ionic strength was 0.006–0.008 M; under these conditions of pH and ionic strength, the reduction potential of iron hexacyanide was taken to be 380 mV (Hanania et al., 1967).

UV–Visible Spectroscopy. The equilibrium constants for formation of the alkaline conformers of the ferricytochrome *c* variants were determined from the pH dependence of the 695-nm band as described by Cutler et al. (1989).

NMR Spectroscopy. Samples of the ferricytochromes were prepared for NMR by exchanging H_2O for D_2O by centrifugal ultrafiltration in Centricon-10 devices (Amicon). Three cycles of concentration and dilution with D_2O were used to exchange NH protons for deuterons. Final protein concentrations were ~1 mM. Samples of ferrocytochrome *c* were obtained by reduction of ferricytochrome samples prepared as described above with stoichiometric amounts of sodium ascorbate. pH values were monitored with a Radiometer Model PHM 82 pH meter equipped with a Russell glass electrode and adjusted by the addition of NaOD and DCl. Quoted pH values are direct meter readings uncorrected for the small isotope effect and are, therefore, designated by pH*. ^1H NMR spectra were recorded at 25 °C with a JEOL GX-400 NMR spectrometer. Resolution-enhanced spectra were obtained with the standard Gaussian multiplication routine of the JEOL PLEXUS program. Nucler Overhauser enhancement (NOE) spectra were obtained as described previously (Moore & Williams, 1984). 1,4-Dioxane was used as an internal standard, but all chemical shifts are quoted in parts per million (ppm) downfield from the methyl resonance of 2,2-dimethyl-2-silapentane-5-sulfonate.

Circular Dichroism Spectroscopy. Protein samples were oxidized prior to use by the addition of $NH_4[Co(dipicolinate)_2]$ as described previously (Rafferty et al., 1990). The ultraviolet CD spectra of wild-type and variant cytochromes *c* were obtained with a Jasco Model J-600 spectropolarimeter controlled by a microcomputer at the National Research Council (Ottawa, Ontario).

RESULTS

Electrochemical Studies. As observed for other iso-1-cytochrome *c* variants (Cutler et al., 1989), the midpoint potentials of the variants studied here are markedly pH-dependent, decreasing almost continuously with increasing pH (Figure 1). The solid lines in Figure 1 have been generated

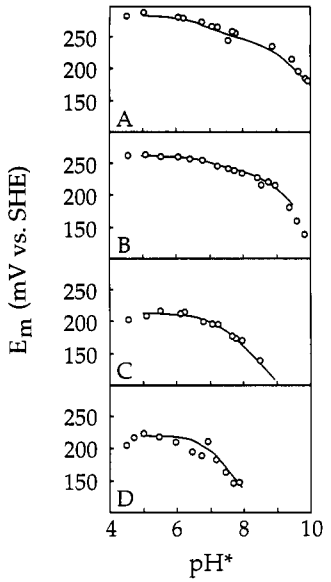

FIGURE 1: Dependence of reduction potentials (vs SHE) of yeast iso-1-cytochrome c variants on pH (25 °C, $\mu < 0.01$ M). The solid lines are theoretical curves generated with eq 1 and the experimentally determined pK_a values found in Table I. (A) Wild-type iso-1-cytochrome c; (B) Tyr48Phe variant; (C) Tyr48Phe/Trp59Phe variant; (D) Arg38Ala/Tyr48Phe/Trp59Phe variant.

Table I: Reduction Potentials and Redox-Linked pK Values of Yeast Iso-1-cytochrome c Variants (mV vs SHE)[a]

cytochrome	E	$E_{m,7}$	ΔE	pK_{O1}	pK_r	pK_{O2}
wild-type	285	265	0	7.26	6.64	8.8
Arg38Ala	240	233	45	7.23	6.97	8.3
Tyr48Phe	263	252	22	7.23	6.86	8.6
Tyr48Phe/Trp59Phe	215	202	70	(7.23)	(6.86)	7.6
Arg38Ala/Tyr48Phe/Trp59Phe	220	198	65	(7.23)	(6.86)	7.0

[a] pK_{O1} and pK_r are the ionization constants for His-39 in ferro- and ferricytochrome c, respectively; pK_{O2} is the apparent pK for the alkaline transition. pK_{O1} and pK_r were determined for all proteins by ^{1}H NMR except for proteins with multiple substitutions. For these proteins, the values for the Phe-48 variant were assumed (values in parentheses). The pK_{O2} values were determined by titration of the 695-nm absorbance. The uncertainty in midpoint potentials is ±3 mV, and pK values are accurate to ±0.1 pK unit.

from the relationship (Moore et al., 1984):

$$E_m = E + \frac{RT}{nF} \ln\left[\frac{[H^+]^2 + K_r[H^+]}{[H^+]^2 + K_{O1}[H^+] + K_{O1}K_{O2}}\right] \quad (1)$$

In this equation, K_{O1} and K_r are the equilibrium constants for protonation of a redox-linked titratable group [probably His-39 (Robinson et al., 1983; Cutler et al., 1989)] in the reduced and oxidized cytochrome, respectively, and K_{O2} is the equilibrium constant for formation of the alkaline conformation of ferricytochrome c. The alkaline form of cytochrome c is known to be reduced by dithionite (Greenwood & Wilson, 1965) but not by ascorbate (Wilson & Greenwood, 1971), which is consistent with the ~−200 mV midpoint reduction potential observed for this conformation of the protein (Barker & Mauk, 1992). Values for the redox-linked pK values were obtained from spectroscopic studies (Moore et al., 1984; Cutler et al., 1989) and are given in Table I.

NMR Spectroscopy. The NMR spectra of the Tyr48Phe and Tyr48Phe/Trp59Phe variants are little altered from those of the wild-type protein (Thurgood et al., 1990). 2D-COSY spectra have been used to obtain resonance assignments for 25 spin systems of these variant cytochromes, including the Phe-59 spin system, and a comparison of the chemical shifts

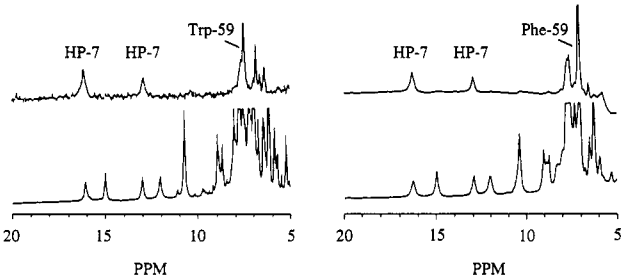

FIGURE 2: NMR spectra of wild-type ferricytochrome c (left) and Tyr48Phe/Trp59Phe ferricytochrome c (right) (pH* 6, 25 °C). The lower spectra are the regions of the conventional spectra, and the upper spectra are regions of the NOE difference spectra obtained after irradiation of the heme methyl-8 resonance at ~35 ppm for 0.5 s. HP-7 indicates the β-proton resonances of heme propionate-7.

has been carried out (Thurgood et al., 1990). The major spectral differences between the Tyr48Phe variant and the Tyr48Phe/Trp59Phe variant are a result of differences in the size and shape of the ring current associated with the residue at position 59. This finding was indicated by the chemical shift analysis and by the following NOE experiment. A critical NMR indicator of the spatial relationships of heme propionate-7, heme methyl-8, and the side chain of residue 59 is the NOE difference spectrum obtained upon saturation of the heme methyl-8 resonance of ferricytochrome c (Moore & Williams, 1984; Cutler et al., 1989). The NOE difference spectra in Figure 2 show that the Tyr48Phe/Trp59Phe variant has a similar structure in the vicinity of heme propionate-7 to the Trp59Phe protein. This conclusion is substantiated by both the similar chemical shift values of the heme propionate-7 resonances and the appearance of resonances of Phe-59 or Trp-59 in the difference spectra. This result is particularly important for the Tyr48Phe/Trp59Phe protein because it shows that the Phe-59 side chain occupies part of the space vacated by Trp-59. Similar spectra were obtained for the triply substituted Arg38Ala/Tyr48Phe/Trp59Phe variant, showing that in this protein too the structure of the protein around the base of the heme is little altered by the substitutions.

The pH dependence of the NMR spectra of the Tyr48Phe variant was measured to determine the His-39 pK values and to investigate the ionization of the heme propionates of ferricytochrome c. The procedure followed was the same as that described by Cutler et al. (1989) for position 38 variants of iso-1-cytochrome c. The His-39 pK values were the same, within experimental error, as those of the wild-type protein (Table I). As with the wild-type cytochrome c and the position 38 variants, the heme propionates did not ionize over the pH range 5–8.

The pH dependence of the Phe-59 variants was monitored by NMR over the pH range 5–7. The heme propionates do not ionize in this range. Because of the low value of pK_{O2} and the increased autooxidizability of these variants, neither the ferricytochrome nor the ferrocytochrome His-39 pK values could be determined reliably. However, the good correlation between the experimental data for the reduction potential pH dependence and the theoretical curve calculated assuming the replacement of Trp-59 by Phe does not perturb the His-39 pK values supports the idea that His-39 is not directly affected by the substitutions.

Circular Dichroism Spectroscopy. The CD spectra of wild-type iso-1-ferricytochrome c and three variants are shown in Figure 3. The two negative bands centered at 280 and 290 nm that are observed in the spectra of the wild-type protein and the Tyr48Phe variant are lacking from the spectra of the two variants in which Trp-59 has been replaced by Phe.

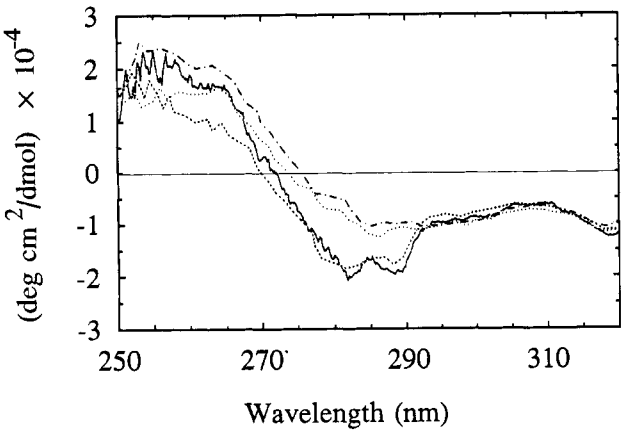

FIGURE 3: Circular dichroic spectra of wild-type and variant forms of yeast iso-1-ferricytochromes *c* [pH 6 (20 mM sodium phosphate buffer), 25 °C]. (Top) Near-UV region (250–320 nm): wild-type (—); Trp59Phe (···); Tyr48Phe (- - -); Tyr48Phe/Trp59Phe (– · –). (Bottom) Soret region.

Similarly, the intensity of the negative Cotton effect seen in the Soret region (data not shown) of the oxidized cytochromes is substantially less for the variant cytochromes lacking a tryptophanyl residue at position 59.

Yeast iso-1-cytochrome *c* possesses four phenylalanyl residues, five tyrosyl residues, one tryptophanyl residue, and two thioether bonds, all of which contribute to the near-UV CD spectrum of the protein (250–330 nm). Interpretation of this region of the spectrum is further complicated by the optically active heme transitions that occur between 240 and 300 nm (Urry, 1967). The vibronic transition bands of tyrosyl residues produce CD maxima between 275 and 282 nm, while phenylalanyl side chains produce CD bands between 255 and 270 nm (Strickland, 1974). The CD spectrum of wild-type ferricytochrome *c* consists of a number of small positive bands centered around 256 nm, a broad positive band centered around 264 nm, and two broad negative bands at 282 and 289 nm, respectively. The broad positive CD transition around 263 nm has been attributed to porphyrin transitions while their rotary strengths are dependent on the immediate environment of the heme (Urry, 1967). The large reduction in the rotary strength of the two negative CD bands at 282 and 289 nm in the variants lacking Trp-59 suggests that these bands are attributable to this residue. This assignment is further substantiated by the NOE experiments described from which it is clear that replacement of Trp-59 by Phe does not result in significant perturbation in the structure of this region of the protein that might otherwise account for changes in the near-UV CD spectrum. Similarly, the decreased intensity of the negative Cotton effect in the Soret region of ferricytochrome *c* variants lacking Trp-59 suggests that this residue also contributes to the CD spectrum in this region as well, though the contribution is less distinctive.

DISCUSSION

The relative positions of Arg-38, Tyr-48, and Trp-59 in the three-dimensional structure of yeast iso-1-ferrocytochrome *c* (Louie & Brayer, 1990) are shown in Figure 4. As can be seen in this diagram, this region of cytochrome *c* involves a number of hydrogen-bonding interactions between these residues, internally-bound water molecules, and heme propionate-7. As the charged guanidino group of Arg-38 is not accessible to bulk solvent and the two internally-bound water molecules participate in an internal hydrogen-bonding network, the environments of heme propionate-7, Arg-38, and

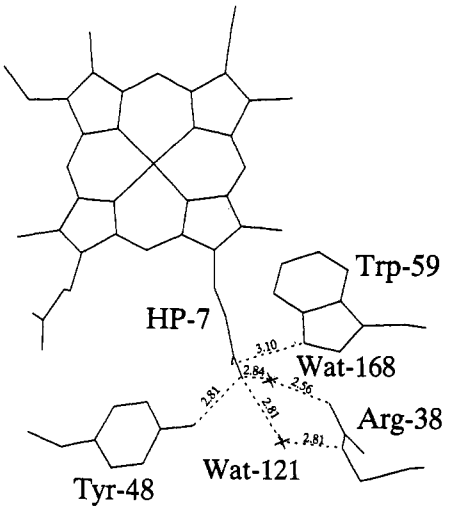

FIGURE 4: Environment of heme propionate-7 in the three-dimensional structure of yeast iso-1-ferrocytochrome *c* (Louie & Brayer, 1990). The water molecules are shown as asterisks centered on the positions of the oxygen atoms. The distance from the heme iron to the nearest atom of the His-39 imidazole ring (C5) (not shown) is 17.4 Å. The residues modified by site-directed mutagenesis in the present study are indicated.

the other hydrogen-bonding residues considered here have aroused the interests of several investigators over the past few years. In particular, the protonation status of heme propionate-7 has attracted considerable attention (Moore et al., 1984; Churg & Warshel, 1986). Following our initial investigation of the role of Arg-38 in determining the pK_a of heme propionate-7 (Cutler et al., 1989), it seemed likely that if the pK_a of this group is decreased below the range of stability of the native conformation of the protein, then the residues selected for study in the current investigation (Figure 4) are likely to make major contributions to this behavior. To assess this possibility, the present study was designed to eliminate the hydrogen-bonding interactions between heme propionate-7 and the amino acid residues constituting its local environment and thereby increase its pK_a into a range of pH where its titration could be detected by NMR spectroscopy and by its effect on the midpoint reduction potential of the heme iron center. The NMR and CD data indicate that the substitutions are not accompanied by substantial conformational changes.

In all of the variant proteins studied here, the oxidized forms of the variant cytochromes are more stable than the corresponding reduced forms compared to the wild-type protein, and, hence, their midpoint reduction potentials are lowered. The interactions between heme propionate-7 and the amino acid residues normally contributing to its local environment withdraw negative charge from the propionate group and thereby destabilize the positive charge on the heme iron. Elimination of the hydrogen bond between Tyr-48 and the heme propionate in the Tyr48Phe variant reduces the degree of charge withdrawal from the propionate with the result that the Fe(III) heme iron is stabilized relative to the wild-type protein. The loss of this single hydrogen bond results in a 22-mV drop in the reduction potential, which is equivalent to a ΔG of –2.12 kJ mol^{-1}. The actual contribution of this H-bond may be slightly different from this value according to Kassner's hypothesis (1972), because a decrease in the polarity of the side chain should lead to an increase in the Born solvation energy of the heme charge. This effect, however, will be much smaller than the contribution of the hydrogen bond.

Comparison of the electrochemical properties of the variants in which two or three residues near heme propionate-7 have been replaced with those of proteins with single substitutions

illustrates the subtlety of this region of the protein. For example, although replacement of Tyr-48 by Phe lowers the midpoint reduction potential by 22 mV and the additional replacement of Trp-59 by Phe lowers the midpoint potential approximately 50 mV further, introduction of a third replacement by substituting Ala for Arg-38, which by itself lowers the potential by 45 mV, results in no further change in the potential. Clearly, the naive possibility that these substitutions might exhibit additive effects on the electrochemical properties is not observed. This finding presumably arises from mutation-induced modulation in the dielectric environment of heme propionate-7 related to critical changes in solvation that are difficult to quantify in a rigorous and satisfactory manner.

The present experiments were based on the assumption that heme propionate-7 titrates below the range of pH over which the native conformation of the protein is stable. Consequently, the amino acid substitutions introduced in the variants studied were expected to increase the pK_a of heme propionate-7. Our failure to observe this effect could arise in two ways. The simpler explanation is that the electrostatic properties of this propionate group are insufficiently perturbed by the modification we have introduced to result in any perceptable change in its behavior. On the other hand, if the pK_a of heme propionate-7 is abnormally high, then our modifications could be argued to have simply elevated this pK_a even higher. In this case, introduction of additional basic residues near heme propionate-7 would presumably be required in an attempt to stabilize the deprotonated form of the carboxyl group. Substitutions of this type are more complex to design as they involve more extreme structural modifications than those used in the present study and may result in a significant destabilization of the native conformational state of the cytochrome. Also, they are not supported by the studies on cytochromes c_2 described in the introduction. We conclude that the reduction potential of cytochrome *c* is affected by a complex interplay of structural factors in the vicinity of heme propionate-7 that modulate the dielectric environment of this functional group and thereby affect the differential stability of the Fe^{2+} and Fe^{3+} forms of the protein.

ACKNOWLEDGMENT

We thank Dr. Gordon Willick of the National Research Council of Canada (Ottawa, Ontario) for access to and assistance in the operation of the spectropolarimeter used in this work and Robert Maurus for preparation of Figure 4.

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